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Effect of a Fullerene Water Suspension on Bacterial Phospholipids and Membrane Phase Behavior

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Several fullerene-based nanomaterials generate reactive oxygen species that can damage cells. In this study, we investigated the effect of buckminsterfullerene (C_{60}) introduced as colloidal aggregates in water (nC_{60}) on bacterial membrane lipid composition and phase behavior. *Pseudomonas putida* (Gram-negative) and *Bacillus subtilis* (Gram-positive) responded to nC_{60} by altering membrane lipid composition, phase transition temperature, and membrane fluidity. *P. putida* decreased its levels of unsaturated fatty acids and increased the proportions of cyclopropane fatty acids in the presence of nC_{60}, possibly to protect the bacterial membrane from oxidative stress. Fourier transform infrared spectroscopy measurement of intact bacterial cells showed slightly increased phase transition temperatures (T_m) and increased membrane fluidity for cells grown in the presence of high, growth-inhibiting concentrations (0.5 mg L^{-1}) of nC_{60}. *B. subtilis* responded to a low dose of nC_{60} (0.01 mg L^{-1}) by significantly increasing the levels of *iso*- and *anteiso*-branched fatty acids (from 5.8 to 31.5% and 12.9 to 32.3% of total fatty acids, respectively) and to a high, growth-inhibiting concentration of nC_{60} (0.75 mg L^{-1}) by increasing synthesis of monounsaturated fatty acids. In contrast to *P. putida*, *B. subtilis* response was a decrease in T_m and an increase in membrane fluidity. These findings represent the first demonstrated physiological adaptation response of bacteria to a manufactured nanomaterial, and they show that response in lipid composition and membrane phase behavior depends on both the nC_{60} concentration and the cell wall morphology.

Introduction

With the increasing use of nanomaterials in commercial products, interest in the broader impact of nanomaterials on ecosystem health has grown steadily (*1*). It is critically important that the environmental risk of nanomaterials is well defined to avoid unintended damage.

Fullerenes are one type of manufactured nanomaterials that are lipophilic (*1*, *2*) and are able to partition into cell membranes (*3*). They can also be toxic to rodents (*4*−*6*). There are numerous reports of the ability of C_{60} and C_{70} fullerenes to cleave DNA, inactivate viruses, and kill tumor cells (*7*). C_{60}, a highly hydrophobic compound, can partition into water either through functionalization to produce a hydrophilic derivative or by making a stable fullerene water suspension. These fullerene water suspensions (nC_{60}) can be produced using transitional solvents or by long-term stirring of C_{60} powder in water (*8*−*10*). All of these nC_{60} suspensions have strong antibacterial properties (*11*, *12*). They have also been demonstrated to be toxic to fish and human cell lines (*11*, *13*). A recent fish toxicity study showed that lipid peroxidation in the brain increased significantly in fish exposed to 0.5 ppm of nC_{60} (*2*). A follow-up study showed that the peroxisomal lipid transport protein PMP70 was down-regulated in the fathead minnow, indicating alterations in the acyl-CoA pathways (*14*). It is unclear whether lipid peroxidation is an important toxicity mechanism in bacteria, since bacterial lipids are mainly monounsaturated and thus unreactive to the lipid peroxidation chain reaction (*15*, *16*).

Reactive oxygen species (ROS) have been implicated in the antibacterial mechanism of one type of fullerene water suspension (*17*). In another study of nC_{60} with bacteria, toxicity was not affected by the presence or absence of light that would be needed to stimulate photocatalytic ROS production by nC_{60} (*18*). In addition to light, oxygen is a critical precursor to ROS generation. In previous research, nC_{60} inhibited the growth of both *Escherichia coli* and *Bacillus subtilis* under anaerobic and fermentative conditions where O_2 was absent (*19*). These lines of evidence indicate that photocatalyzed ROS production either plays no role or that it is not the sole antibacterial mechanism associated with nC_{60}.

The ability of nC_{60} to form stable water suspensions makes it relatively accessible to biological systems (*20*). Since little is known regarding the interaction of nC_{60} with living systems, investigating its effect on bacteria (and the associated adaptation mechanisms) is important not only because of the potential impact on microorganisms that serve as the basis of the food chain and as primary agents for biogeochemical cycles but also because similar nanomaterials might find applications in controlling microbial pathogens in drinking water to protect public health. In this study, we performed a comparative study on Gram-negative (*Pseudomonas putida*) and Gram-positive (*B. subtilis*) bacterial responses to sublethal concentrations of nC_{60}. This is the first investigation of how bacteria respond physiologically to the presence of a manufactured nanomaterial. Specifically, membrane dynamics of bacteria grown in the presence or absence of nC_{60} are assessed based on lipid compositional changes and Fourier transform infrared spectroscopy (FTIR) measurement of intact bacterial cells.

Materials and Methods

Bacterial Growth Conditions and Exposure to nC_{60}. *P. putida* F1 and *B. subtilis* CB310 (courtesy of Dr. Charles Stewart, Rice University, Houston, TX) were selected for the comparative study of bacterial response to exposure of nC_{60} fullerene. Cells were maintained on Luria broth or agar plates, but all experiments were performed in a minimal Davis medium (MD), which has 90% less potassium phosphate

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than regular Davis medium, to avoid salt-induced precipitation of nC_{60} suspensions (*19*). Cells were inoculated into MD to an OD_{600} of 0.01. The minimal inhibitory concentration (MIC) of nC_{60} to each bacterium was determined using Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) methodology as described by Lyon et al. (*18*). The number of viable bacteria was assessed by plating an aliquot of the bacteria onto LB plates, counting the number of colonies that had grown overnight at 37 °C, and calculating the number of colony forming units per milliliter of culture (cfu mL^{-1}).

The bacteria were exposed to either MD without nC_{60} (negative control), with low levels of nC_{60} (0.01 mg L^{-1}), or with high growth-inhibiting levels of nC_{60} (0.5 mg L^{-1} for *P. putida* and 0.75 mg L^{-1} for *B. subtilis*) while shaking or stirring overnight for 14 h in a 37 °C incubator. The amount of nC_{60} added to achieve the high level was equal to the MIC to ensure cell viability after exposure and to investigate the sublethal physiological response. The levels differ for *P. putida* and *B. subtilis* as these bacteria are susceptible to nC_{60} at different concentrations. For phospholipids fatty acid (PLFA) analysis, the cells were harvested by centrifugation at 10000*g* for 10 min to yield at least 100 mg of wet cell mass per sample. The bacteria were inoculated into the different samples to a final OD_{600} of 0.01, and the total volume of the sample was adjusted to achieve the desired cell concentration.

Manufacturing nC_{60}. The nC_{60} was produced as described by Fortner et al. (*21*). Briefly, 100 mg of 99.5% pure C_{60} (MER Corporation, Tucson, AZ) were stirred overnight in 4 L of nitrogen-sparged, spectra-analyzed tetrahydrofuran (THF) (Fisher Scientific, Houston, TX). After undissolved particles were removed by filtration through a 0.22 μm membrane, 500 mL of the C_{60}−THF solution was stirred vigorously while an equal volume of Milli-Q (Millipore, Billerica, MA) water was added at a rate of 1 L min^{-1}. The THF was evaporated away using a Büchi Rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) while heating at 65 °C. The nC_{60} was concentrated by evaporating excess water in the Büchi Rotavapor to a final concentration of 11 mg/L C_{60}. The concentrated suspensions were filtered-sterilized through a 0.22 μm cellulose syringe filter (Fisher Scientific) to obtain particles ranging in size from 50 to 200 nm, with the mean diameter of 95 nm as measured using a dynamic light scattering device (Brookhaven Instrument Corporation, Holtsville, NY).

Lipid Extraction. Total lipids were extracted with a modified Bligh and Dyer extraction method (*21*). Cell pellets were extracted for lipids in test tubes filled with 11.5 mL of methanol, dichloromethane (DCM), and phosphate buffer (2:1:0.8) extraction solution. The extraction mixture was allowed to stand overnight in darkness at 4 °C. The lipids were then partitioned by adding DCM and double-distilled and deionized water such that the final ratio of DCM−methanol−water was 1:1:0.9. The upper aqueous phase was discarded, and the lower organic phase was then decanted through a glass fiber filter into a test tube. The solid residue retained on the filter was washed with 3 × 1 mL dichloromethane. The total lipid extract was dried under a gentle stream of nitrogen and was once again dissolved in methanol. Total lipids were separated into different lipid classes using miniature champagne columns (Supelco Inc., Bellefonte, PA). Neutral lipids, glycolipids, and phospholipids were eluted with 4 mL of chloroform, acetone, and methanol, respectively (*21*). Ester-linked phospholipid fatty acids were subjected to a mild alkaline trans-methylation procedure to produce fatty acid methyl ester (FAME). Part of the FAME was subjected to procedure of Kleiman and Spencer (1973) by reacting with BF3/methanol (500 μL, 65 °C for 25 min) and then with 60 μL of *N,O*-bis(trimethylsilyl)-trifloroacetamide (65 °C for 1 h). Opening of the epoxy ring forms methoxy-hydroxy derivatives of epoxy fatty acids (*22*). Neutral and glycolipids were checked for oxygenated (oxo- and epoxy-) fatty acids by first saponifying the fractions with 6.0 M HCl:methanol (1.0:0.85) and then reacting with BF3/methanol and *N,O*-bis(trimethylsilyl)-trifloroacetamide. Olive oil fried at about 200 °C for 5 min was subjected to the same lipid extraction and derivatization procedures as described above to serve as the positive control for oxygenated fatty acids.

Analysis of Fatty Acid Methyl Esters by Gas Chromatography/Mass Spectrometry (GC/MS). FAMEs were analyzed on an Agilent 6890 GC interfaced with an Agilent 5973N mass selective detector. Analytical separation of the compounds was accomplished using a 30 m × 0.25 mm i.d. DB-5 MS fused-silica capillary column (J&W Scientific, Folsom, CA). The column temperature was programmed from 50 °C to 120 °C at 10 °C/min, then to 280 °C at 3 °C/min. Individual compounds were identified from their mass spectra. Double-bond position and geometry of monounsaturated fatty acids were determined by using methods described by Dunkleblum et al. (*23*). Response factors were obtained for fatty acids by injecting calibration standards at five different concentration levels (0.05, 0.1, 0.5, 1.0, and 2.0 ng μL^{-1}). Concentrations of individual compounds were obtained based on the GC/MS response relative to that of an internal standard ($C_{18:0}$ fatty acid ethyl ester). Method blanks were extracted with each set of samples and were assumed to be free of contamination if chromatograms contained no peaks. Fatty acids are designated by the total number of carbon atoms to the number of double bonds (i.e., a 16-carbon alkanoic acid is 16:0). The position of the double bond is indicated with a Δ number closest to the carboxyl end of the fatty acid molecule with the geometry of either *c* (*cis*) or *t* (*trans*). The cyclopropyl group is indicated with *cy*.

Fourier Transform Infrared Spectroscopy Measurements. The temperature-dependent vibrational frequency of the CH_2-stretching in lipids of intact bacterial cells was measured using a Nic-Plan IR microscope attached to a Magna 750 FTIR spectrometer (Nicolet). A thin layer of bacterial cell pellets were spread on a polished aluminum pan and fitted into a differential scanning calorimetry (DSC) stage (Linkam) with temperature range of −196 to 600 °C. The DSC stage was continuously purged with pure N_2 gas during the measurement. The samples were cooled to −40 °C and slowly heated at a rate of 1 °C/min. A spectrum was recorded every 2 °C with 4 min of equilibration. Each spectrum contains the average of 64 scans from 4000 to 650 cm^{-1} at 0.5 cm^{-1} resolution. Background scans were performed on the blank aluminum surface at each temperature and subtracted from the sample spectra. Membrane fluidity and phase transitions were monitored by observing the position of the CH_2 asymmetric stretching band at ∼2926 cm^{-1}. The first derivative of the frequency versus temperature curves was used to determine the transition temperature (T_m), which is given as the point of inflection coinciding with the gel−fluid transitions (*28*).

Results

Bacterial Behavior. The MIC of nC_{60} for *B. subtilis* was between 0.5 and 0.75 mg L^{-1}, whereas that for *P. putida* was between 0.25 and 0.5 mg L^{-1}. Bacteria exposed to the high nC_{60} concentrations (0.75 mg L^{-1} for *B. subtilis* and 0.5 mg L^{-1} for *P. putida*, which are equivalent to the MICs) did not grow overnight. As expected, nC_{60} exerted a bacteriostatic effect consistent with previous studies (*18*). These cultures also experienced a 1 order of magnitude decrease in viability, as assessed by plate counts, from approximately 10^7 to 10^6 cfu mL^{-1}. In contrast, when exposed to the low nC_{60} concentration (0.01 mg L^{-1}), both bacteria grew after 14 h to the same optical density as their corresponding nC_{60}-free controls, from OD_{600} of 0.01 to 0.15 for *B. subtilis* and from OD_{600} of 0.01 to 0.05 for *P. putida*.

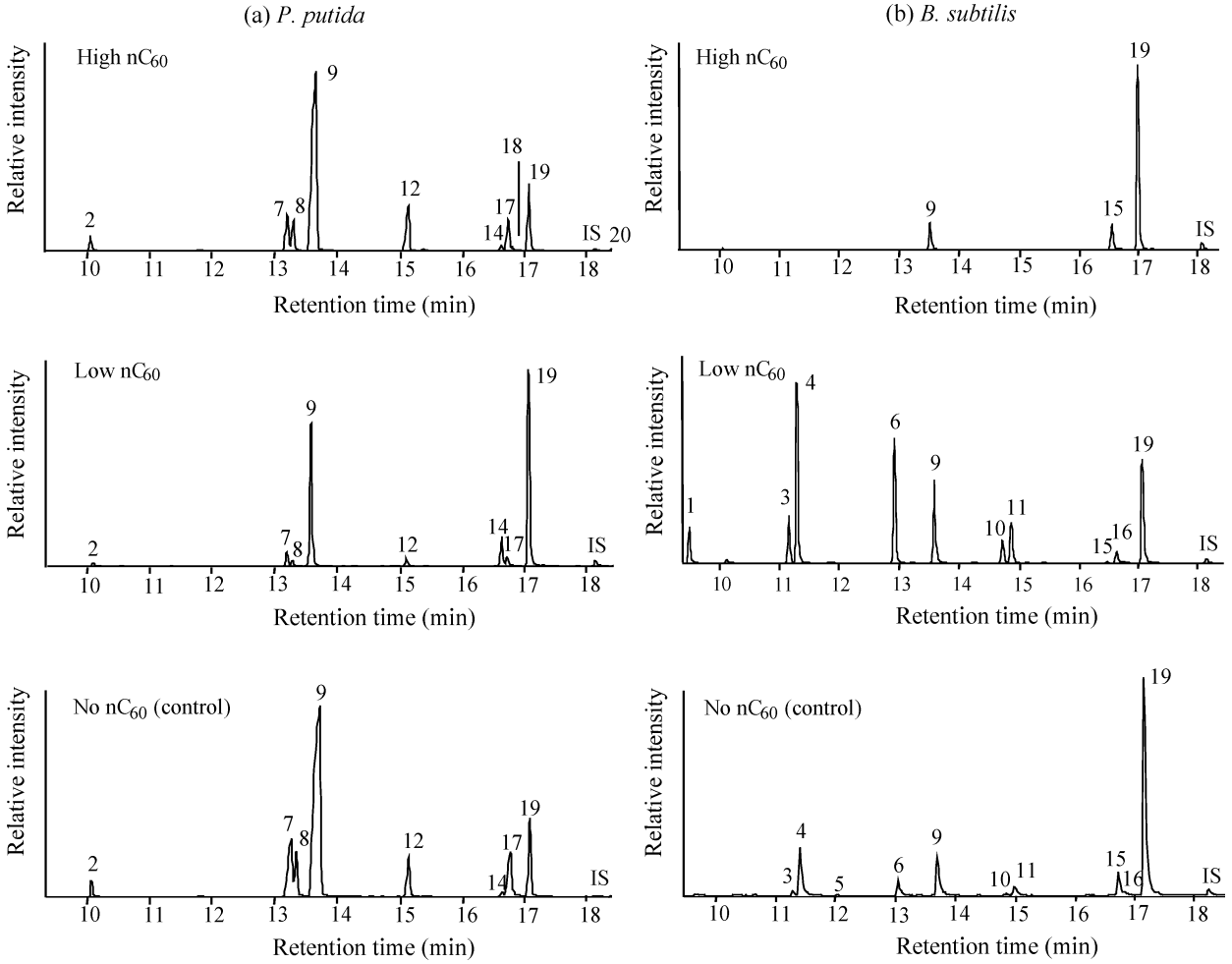

FIGURE 1. GC/MS chromatograms of fatty acids isolated from (a) *P. putida* and (b) *B. subtilis* grown in the presence or absence of nC_{60}. See Table 1 for fatty acid identification and concentrations.

Phospholipid Fatty Acids. Fatty acids identified in *P. putida* cells were similar to those reported before (*24*) (Figure 1). Saturated, monounsaturated and cyclopropane fatty acids isolated from *P. putida* cell grown without nC_{60} (control) constitute 36.2, 60.1, and 3.6% of the total fatty acids, respectively (Table 1). *P. putida* grown in the presence of low concentrations of nC_{60} (0.01 mg L^{-1}) showed increased proportions of saturated fatty acids (63.1%) and reduced synthesis of monounsaturated fatty acids (32.9%). This is in agreement with previous observations that apolar organics cause a decrease in the amount of unsaturated fatty acids (*25*). The proportions of cyclopropane fatty acids increased slightly from 3.6 to 4.0%, and the *trans*/*cis* ratio of 16:1Δ^9 increased from 0.46 to 0.50. These changes were more pronounced in *P. putida* cells grown in the presence of high concentrations of nC_{60} (0.5 mg L^{-1}), which experienced a net increase of 16% in the proportion of *cy*17:0 fatty acids, a concurrent decrease in *cis*-monounsaturated fatty acid (16:1Δ^9*c*), and an increase in *trans*-monounsaturated fatty acids (Table 1), resulting in an increase in *trans*/*cis* ratio of 16:1 Δ^9*c* from 0.46 to 0.80.

The baseline fatty acid profile of *B. subtilis* was similar to that reported by Kaneda (*26*) (Figure 1). Exposure to nC_{60} at 0.01 mg L^{-1} resulted in markedly increased levels of *iso*- and *anteiso*-branched fatty acids (31.5 and 32.3% of total fatty acids, respectively), while the percentage of saturated (28.1%) and unsaturated fatty acids (8.1%) decreased substantially (Table 1). When cells were exposed to higher doses of nC_{60} (0.75 mg L^{-1}), the proportion of *iso*- and *anteiso*-branched fatty acids nearly disappeared (0.1%), whereas the proportions of monounsaturated fatty acids (*cis* and *trans* 18:1Δ^9) increased significantly (from 24.2 to 30.2%). Such a high level of monounsaturated fatty acids has not been observed before in any *B. subtilis* strains.

Oxygenated (oxo- or epoxy-) fatty acids were not detected in *P. putida* or *B. subtilis* grown in the presence or absence of nC_{60}. For the positive control (fried olive oil), 9,10-epoxystearate was detected, which constituted about 2% of the total fatty acids (data not shown).

Membrane Lipid Phase Behavior: FTIR. Membrane phase behavior of intact bacterial cells was assessed by FTIR. Infrared spectroscopy measures the low-energy transitions between vibration levels generated by characteristic motions (e.g., stretching) of different chemical bonds in the lipid molecule (*27*). The increase in vibration frequency denotes an increase in membrane fluidity as the sample passes through the gel−fluid transition with increasing temperature due to the sequential melting of lipids in the membrane (*27*, *28*). Phase transitions taking place over a broader temperature range indicates a less cooperative melting process (*28*). Figure 2 shows the temperature dependence of the asymmetric methylene stretching vibration frequency in intact *P. putida* and *B. subtilis* cells. The gel−liquid crystalline phase transitions are evident in all of the cells grown in the presence or absence of nC_{60}. The *B. subtilis* cells of the control showed a broader phase transition over a wide temperature range. The phase transition temperature is evident at ∼−2.5 °C (Figure 2a), accompanied by a relatively small increase in frequency (1.2 cm^{-1}) at the transition. Cells grown in the presence of low (0.01 mg L^{-1}) and high nC_{60} concentrations

TABLE 1. Fatty Acid Composition (Mole Percentage of Total Fatty Acids) of *P. putida* and *B. subtilis* Grown without nC_{60} (Control) and with Low (0.01 mg L^{-1}) and High (0.5 mg L^{-1} for *P. putida* and 0.75 mg L^{-1} for *B. subtilis*) Concentrations of nC_{60}[a]

		P. putida			*B. subtilis*		
peak	fatty acid	control	low	high	control	low	high
1	*i*14:0	0.0	0.0	0.0	0.0	5.1	0.0
2	14:0	1.0	0.7	1.6	0.0	0.0	0.0
3	*i*15:0	0.0	0.0	0.0	1.1	6.8	0.0
4	*a*15:0	0.0	0.0	0.0	12.0	24.6	0.2
5	15:0	0.0	0.0	0.0	0.2	0.0	0.0
6	*i*16:0	0.0	0.0	0.0	3.8	15.6	0.1
7	16:1$\Delta^9 c$	27.5	7.6	16.3	0.0	0.0	0.0
8	16:1$\Delta^9 t$	12.4	3.8	12.3	0.0	0.0	0.0
9	16:0	30.9	27.7	29.6	11.2	13.0	10.5
10	*i*17:0	0.0	0.0	0.0	0.5	3.9	0.1
11	*a*17:0	0.0	0.0	0.0	0.9	7.2	0.0
12	*cy*17:0	3.5	4.1	17.0	0.0	0.0	0.0
13	17:0	0.0	0.0	0.0	0.0	0.0	0.0
14	18:1	0.3	16.0	1.7	0.0	0.2	0.0
15	18:1$\Delta^9 c$	0.0	0.0	0.0	16.3	6.7	33.7
16	18:1 $\Delta^9 c$	0.0	0.0	0.0	6.1	1.2	0.0
17	18:1$\Delta^{11} c$	19.3	8.1	13.2	0.0	0.0	0.0
18	18:1$\Delta^{11} t$	0.6	0.0	1.3	0.0	0.0	0.0
19	18:0	4.2	32.0	6.5	47.8	15.6	55.2
20	*cy*19:0	0.1	0.0	0.4	0.1	0.0	0.1
	SAFA	36.2	63.1	38.5	57.1	28.1	69.6
	MUFA	60.1	32.9	42.1	24.2	8.1	30.2
	IBFA	0.0	0.0	0.0	5.8	31.5	0.1
	ABFA	0.0	0.0	0.0	12.9	32.3	0.1
	CYFA	3.6	4.0	19.4	0.1	0.0	0.1

[a] SAFA = saturated fatty acids, MUFA = monounsaturated fatty acids, IBFA = *iso*-branched fatty acids, ABFA = *anteiso*-branched fatty acids, CYFA = cyclopropane fatty acids. Peak labeling is indicated in Figure 1.

(0.75 mg L^{-1}) had sharper phase transitions over narrower temperature ranges and higher frequency increases, 1.4 and 2.2 cm^{-1}, respectively.

P. putida cells had very different FTIR patterns (Figure 2b). Cells grown in the presence of low (0.01 mg L^{-1}) nC_{60} concentrations showed a dramatic decrease in the frequency of CH_2 vibration in the gel and in the liquid−crystalline phase; the frequency in the liquid−crystalline phase was even lower than that of the gel phase of the control cells and cells grown at high nC_{60} concentration. This suggests a marked decrease in membrane fluidity, which is also indicated by the increase in saturated fatty acids and decrease in unsaturated fatty acids (Table 1). It is noted that *P. putida* cells grown at low concentrations of nC_{60} had a much larger frequency increase (5.2 cm^{-1}) in both phases than the other two cultures, suggesting an increased in heterogeneity and a decrease in cooperativity of membrane lipids. Cells grown in the presence of 0.5 mg L^{-1} nC_{60} had the highest frequency (2926.2 to 2928.5 cm^{-1}) among all three cultures, reflecting the increased conformation disorder of lipid acyl chains.

Discussion

The tolerance of Gram-negative bacteria to organic solvents has been well studied (e.g., *29, 30*). The most studied are various strains of *P. putida* and *E. coli*. Generally, Gram-negative bacteria respond to organic solvents by altering the relative proportions of saturated and unsaturated fatty acids and fatty acid chain length and by increasing biosynthesis of *trans* fatty acids through *cis*−*trans* isomerization of monounsaturated fatty acids. This study provides the first data on lipid compositional changes and membrane phase behavior of Gram-positive and Gram-negative bacteria exposed to fullerenes. Our results showed that *P. putida* responded differently to different concentrations of nC_{60}. Cells grown in the presence of 0.01 mg L^{-1} nC_{60} had higher levels of saturated fatty acids, lower levels of unsaturated fatty acids, and increased *trans*/*cis* ratios of 16:1Δ^9 compared to the control. The increased synthesis of *trans* unsaturated fatty acids via *cis*−*trans* isomerization has been interpreted as a general defense mechanism employed by microorganisms responding to various stress conditions, including exposure to organic solvents, heavy metals, salts, antibiotics, starvation, temperature, and water potential extremes (*29*, *31*, *32*).

In studying microbial exposure to organic solvents, *cis*−*trans* isomerization has been suggested as a short-term defense mechanism to decrease the penetration of membrane-active compounds through the inner membrane of Gram-negative bacteria (*30*, *32*, *33*). Phospholipids containing *trans* unsaturated fatty acids have a higher phase transition temperature than those containing *cis* unsaturated fatty acids. Therefore, membrane fluidity decreases. This is in agreement with the FTIR spectra which show a substantial decrease in frequency of the CH_2 asymmetrical stretching in cells exposed to low concentration of nC_{60} (Figure 2b). This observation is consistent with previous studies on microbial tolerance to organic solvents (*32*). The dramatic change in the frequency of the CH_2 stretching suggests that the overall conformational order of the membrane acyl chains was substantially altered, possibly by oxidative stress exerted by nC_{60}. In contrast, cells grown at 0.5 mg L^{-1} nC_{60} showed increased membrane fluidity as indicated by the increased CH_2-stretching frequency. Thus, depending on the toxicity and concentration of the toxic compounds present, microbial response may result in decreased or increased membrane fluidity in Gram-negative bacteria. The increased synthesis of *trans* fatty acids in cells grown at high (0.5 mg L^{-1}) concentrations of nC_{60} may be a defense mechanism to decrease the permeability of the lipid bilayer and prevent the membrane from becoming too fluid for optimal growth (*34*−*37*).

What is evidently different from previous bacterial toxicity studies is that the proportions of cyclopropane fatty acids increased consistently with the concentrations of nC_{60} present in the growth medium. This is in contrast to that observed in *P. putida* DOT-T1 cells exposed to toluene where concentrations of cyclopropane fatty acids decreased with toluene exposure (*32*). The biochemical and physiological function of cyclopropane fatty acids in bacteria remains unclear. One plausible hypothesis is that the increased synthesis of cyclopropane fatty acids may be related to their protective ability against oxidation of unsaturated fatty acids by activated oxygen species (*34*, *38*). Grogan and Cronan (*38*) showed that *E. coli* strains that were *cfa*$^-$ (i.e., lacking the cyclopropane fatty acid synthase gene) were more sensitive than isogenic *cfa*$^+$ strains to freeze−thaw treatment, indicating that cyclopropane fatty acids protect bacteria from environmental stress. Presumably, these fatty acids are more stable and less reactive membrane components than are unsaturated fatty acids (*39*) and resist the chemical attack of singlet oxygen (*38*). Fullerenes can be generators of highly reactive singlet oxygen (*40*, *41*). The increased proportion of cyclopropane fatty acids with a balanced decrease of *cis*-unsaturated fatty acids suggests that the conversion of *cis* monounsaturated fatty acids to cyclopropane fatty acids may be a mechanism to protect bacterial cells from membrane oxidation by nC_{60}. Another possibility is that membrane lipid oxidation is not the primary manifestation of oxidative stress from nC_{60}. Indeed, we did not detect any oxygenated fatty acids (oxo- and epoxy-fatty acids) in *P. putida* or *B. subtilis* cells. This could suggest a protective role of cyclopropane fatty acids for Gram-negative bacteria exposed to nC_{60}. Alternatively, it is possible that, following peroxidation of unsaturated fatty acids, lipid peroxides are converted by

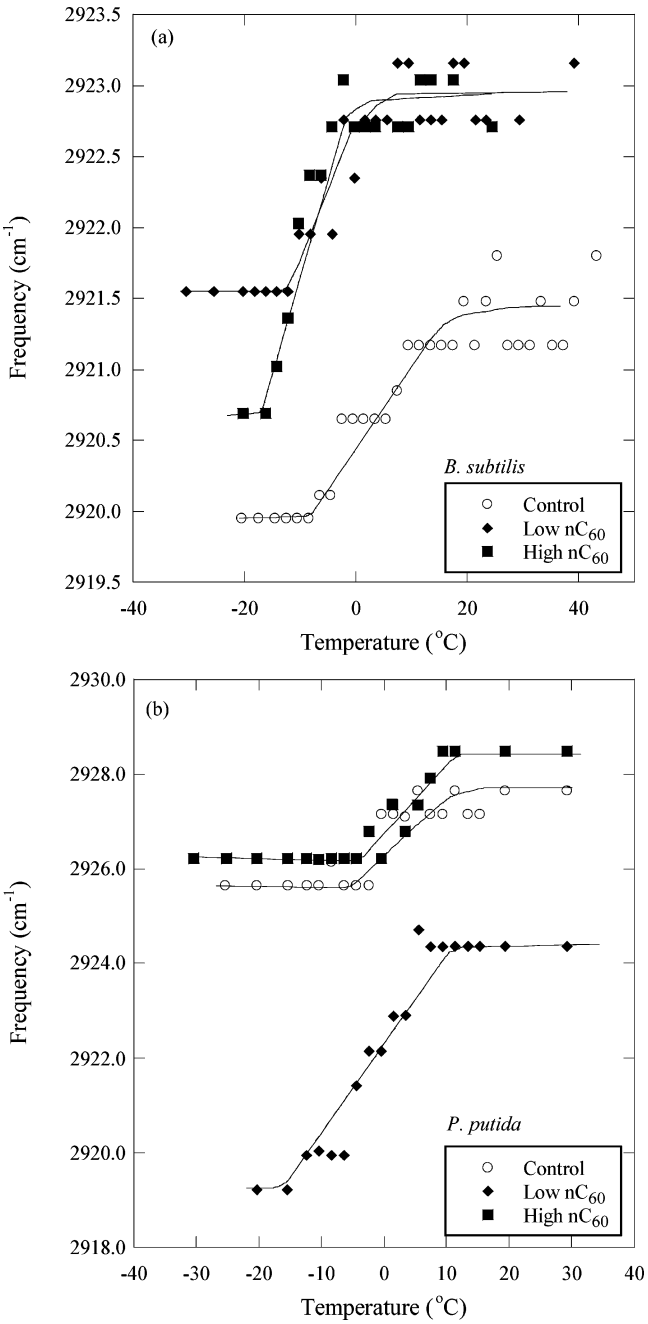

FIGURE 2. Thermotropic response of the CH_2 asymmetrical stretching frequency of intact (a) *P. putida* and (b) *B. subtilis* cells, grown without nC_{60} (control) and with low (0.01 mg L^{-1}) and high concentrations of nC_{60} (0.5 mg L^{-1} for *P. putida* and 0.75 mg L^{-1} for *B. subtilis*).

consecutive reactions of oxidation, rearrangement, and scission into more stable carbonyl compounds such as malondialdehyde (*42*).

Only a few studies have been reported on Gram-positive bacterial responses to toxic chemicals. Our understanding of mechanisms of Gram-positive bacterial response to membrane-active compounds is limited (*43*, *44*). Recently Nielsen et al. (*43*) reported that Gram-positive bacteria *Staphylococcus haemolyticus* was able to tolerate aromatic hydrocarbons (benzene and toluene) by increased synthesis of *iso* and *anteiso*-branched fatty acids and reduced proportions of straight-chain fatty acids. Our results suggest that *B. subtilis* grown in the presence of low concentrations of nC_{60} showed a similar response. When grown in the presence of 0.01 mg L^{-1} nC_{60}, *B. subtilis* showed decreased proportions of saturated and unsaturated fatty acids but sharply increased proportions of *iso* and *anteiso*-branched fatty acids (from 5.8 to 31.5% and 12.9 to 32.3%, respectively). Similar fatty acid compositional changes have been observed in *B. subtilis* subjected to cold shock (*45*) and in other Gram-positive bacteria (six strains of *Staphylococcus aureus* and five strains of *Staphylococcus epidermidis*) grown on toluene (*43*). *Iso*- and *anteiso*-branched fatty acids have the same ability as unsaturated fatty acids to disrupt the close packing of phospholipid acyl chains and lower the temperature of phase transition (*26*). FTIR spectra show that cells grown at low (0.01 mg L^{-1}) and high concentrations (0.75 mg L^{-1}) of nC_{60} had sharper phase transitions over narrower temperature ranges and higher frequency increases. These sharpening transition curves suggest an increase in motional cooperativity between the lipid acyl chains to optimize membrane fluidity in the presence of nC_{60} (*28*). The increase in CH_2 stretching frequency indicates the introduction of conformational disorder (gauche rotations) into the acyl chains of

membrane phospholipids at both low and high concentrations of nC_{60} (*28*). Thus, both FTIR and lipid composition data suggest an increase in membrane fluidity.

When *B. subtilis* cells were exposed to higher doses of nC_{60} (0.75 mg L^{-1}), the proportion of *iso* and *anteiso*-branched fatty acids nearly disappeared (0.1%), whereas the proportions of monounsaturated fatty acids (*cis* and *trans* 18:1Δ^9) increased from 24.2 to 30.2%. Such a relatively high level of monounsaturated fatty acids has not been observed before in any *B. subtilis* strains. This result suggests that unsaturated fatty acids play a more important role in Gram-positive bacterial adaptation to toxic compounds than observed before (*46*), and that Gram-positive bacteria can respond to membrane-active compounds with similar mechanisms to those well-studied constitutive mechanisms used by Gram-negative bacteria. López et al. (*47*) made similar observations; *B. subtilis* responded to hyperosmotic conditions by increased synthesis of 18:1Δ^9. Thus, Gram-negative bacteria can respond differently to the same type of membrane-active compounds under different concentrations, whereas Gram-positive bacteria can respond similarly in fatty acid composition under different stress conditions.

It is generally believed that Gram-negative bacteria tolerate organic solvents better than Gram-positive bacteria because of the protective role of the outer membrane (*48*). However, our FTIR results suggest the opposite trend, with Gram-positive *B. subtilis* showing higher levels of nC_{60} tolerance. This is indicated by the relatively narrow ranges of CH_2-stretching frequency for all cells grown in the presence or absence of nC_{60} (Figure 2). This trend is supported by other studies examining the sensitivity of Gram-positive *B. subtilis* versus the Gram-negative *E. coli* to nC_{60}, which showed that *B. subtilis* was less sensitive than *E. coli* (*18*).

The use of THF as transitional solvent to make nC_{60} should not be a significant confounding factor in the observed antibacterial activity and physiological response. Lyon et al. produced nC_{60} using a variety of methods, including a solvent-free method, and demonstrated the antibacterial activity of each of these differently produced nC_{60}'s (*19*). Recent studies have also concluded that residual dissolved THF was not an influential factor in the toxicity of nC_{60} to zebrafish embryos (*50*) and *Daphnia (51*). This establishes that the antibacterial activity of nC_{60} is independent of residual THF and indicates that the observed changes in membrane composition were primarily due to nC_{60} exposure.

In summary, our results showed that both Gram-negative and Gram-positive bacteria changed their phospholipid composition and therefore the phase transitions assumed by membrane lipids as a result of exposure to sublethal concentrations of nC_{60}. Bacterial response in lipid composition and membrane phase behavior was dependent on both the nC_{60} concentration and the cell wall morphology. The demonstrated effect of nC_{60} on bacterial physiology suggests the potential environmental impact of some nanomaterials and underscores the need for continued research in nanomaterial ecotoxicology.

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